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(54) Title: GRANULINS FROM LEUKOCYTES

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GRANULIN A      DVKCDMEVS-CPDGYTCCRLQSGAWG-CCPFTQAVCCEDHIHCCPAGFTCDTQKGTCE
GRANULIN B       VMCPDARSRCPDGSTCCELPSGKYG-CCPMPNATCCSDHLHCCPQDTVCDLIQSKCL
GRANULIN C       VPCDNVSS-CPSSDTCCQLTSGEWG-CCPIPEAVCCSDHQHCCPQRYTCVAEGQ-CQ
GRANULIN D        IGCDQHTS-CPVGGTCCPSQGGSWA-CCQLPHAVCCEDRQHCCPAGYTCNVKARSCE
GRANULIN E      DVECGEGHF-CHDNQTCCRDNRQGWA-CCPYAQGVCCADRRHCCPAGFRCARRGTKCL
GRANULIN F      AIQCPDSQFECPDFSTCCVMVDGSWG-CCPMPQASCCEDRVHCCPHGAFCDLVHTRCI
GRANULIN G      GGPCQVDAH-CSAGHSCIFTVSGTSS-CCPFPEAVACGDGHHCCPRGFHCSADGRSCF
PARAGRANULIN     TRCPDGQF-CPVA--CCLDPGGASYSCCRPLLD
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(57) Abstract

Novel leukocyte peptides are useful in healing wounds; the peptides are cystine rich and approximately 6 Kda.

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GRANULINS FROM LEUKOCYTES

TECHNICAL FIELD

This invention relates to novel peptides and pharmaceutical formulations containing them; the peptides are useful as inhibitors of keratinocytes.

BACKGROUND ART

It has recently become clear that leukocytes are pedtidergic cells. Neutrophil granules contain large amounts of basic, cystine-rich peptides of 29 to 34 amino acids, that have been variously called defensins (1), coricostatins (2), myeloid-related sequences (3), and cryptidins (4). Some of these peptides are antimicrobial agents at micromolar concentrations (5), and it was initially thought that their only biological activity was in non-oxidative, non-enzymatic, destruction of phagocytosed microorganisms. More recently, however, it has been shown that coricostatins have potential regulatory functions, including the ability to inhibit the action of the hormone adrenocorticotropin on glucocorticoid secretion (2,6,7) and to stimulate nifedipine-sensitive L-type Ca^{2+} channels in villus enterocytes (8). It has also been reported that a human defensin is a monocyte chemotactic agent (9). Other granulocyte-associated peptides have also been shown to have regulatory activities. For example, hemoregulatory peptide 1 is a granulocyte-associated thiol containing pentapeptide, with potent inhibitory actions on myelopoieses (10). Several groups have reported the existence of immunomodulatory or cytokine-like activities associated with neutrophil extracts or supernatants (11,12,13,14). These activities include mast cell degranulation, chemotaxis, and the inhibition of myelopoetic-colony formation. Despite these reports, and the evidence for regulatory actions associated with known granulocyte peptides, few systematic

attempts to characterize the regulatory molecules of the granulocyte seem to have been made. Granulocyte enriched extracts contain several cystine-rich components at levels approximately three orders of magnitude lower than the defensin/corticostatins. From their compositional analysis and chromatographic behaviour these peptides appear unrelated to any known hormone, including the definsin/corticostatins. Granulocyte-derived peptides have potential both as immunoregulatory molecules, and in host resistance.

Granulins are novel candidate growth factors recently discovered in human and rat inflammatory leukocytes (22). Two rat granulin homologs, epithelin 1 and 2, occur in the kidney (23). Epithelin 1, which is probably identical to rat leukocyte granulin (22,23) exhibits activities similar to epidermal growth factor on epithelian cells in vitro (23).

DISCLOSURE OF THE INVENTION

It is an object of this invention to provide novel granulins useful in wound healing.

In particular the invention relates to a family of novel leukocyte-associated peptides that are cystine-rich.

The peptides are approximately 6 Kda and may be cytokines.

In accordance with this invention there is provided Granulin A: DVKCDMEVSCPDGYTCCRLQSGAWGC-CPFTQAVCCEDHIHCCPAGFTCDTQKGTCE, SEQ ID NO: 1.

In accordance with another aspect of the invention there is provided Rat Granulin: EVKCDLEVSCPDGYTCCRLNTGAWG(CCPFSB)AVCCEDHIHCCPAGFTCXTQ, SEQ ID NO: 5.

In accordance with still another aspect of the invention there is provided Granulin C: VPCDXVSSCPSSDTCCOLTSGEHGCCPIPEAVC, SEQ ID NO: 3.

In accordance with yet another aspect of the invention there is provided Granulin D: IGCDQXDTSSCCPDG, SEQ ID NO: 4.

In accordance with a still further aspect of the invention there is provided carp Granulin: VIHCDAATICPDGTICCLSPYGMBGQCCRDGIHCCRHGYHCDSRTTHCL, SEQ ID NO: 6.

In accordance with another aspect of the invention there is provided Granulin B: VMCPDARSRCPDGHTCCELPSGKYGCCPMPNATCCSDHLHCCPQDTVCDLIQSK CI, SEQ ID NO: 2.

In accordance with still another aspect of the invention there is provided Granulin E: Asp Val Glu Cys Gly Clu Gly His Phe Cys His Asp Asp Gln Thr Cys Cys Arg Asp Asn Arg Glu Gly Trp Ala Cys Cys Pro Try Ala Gln Gly Cla Cys Cys Ala Asp Arg Arg His Cys Cys Pro Ala Gly Phe Arg Cys Ala Arg Arg Gly Thr Lys Cys Leu, SEQ ID NO: 7.

In accordance with yet another aspect of the invention there is provided Granulin F: Ala Ile Gln Cys Pro Asp Ser Gln Phe Glu Cys Pro Asp Phe Ser Thr Cys Cys Val Met Val Asp Gly Ser Trp Gly Cys Cys Pro Met Pro Gln Ala Ser Cys Cys Glu Asp Arg Val His Cys Cys Pro His Gly Ala Phe Cys Asp Leu Val His Thr Arg Cys Lle, SEQ ID NO: 8.

In accordance with still another aspect of the invention there is provided Granulin G: Gly Gly Pro Cys Gln Val Asp Ala His Cys Ser Ala Gly His Ser Cys Ile Phe Thr Val Ser Gly Thr Ser Ser Cys Cys Pro Phe Pro Glu Ala Cys Gly Asp Gly His His Cys Cys Pro Arg Gly Phe His Cys Ser Ala Asp Gly Arg Ser Cys Phe, SEQ ID NO: 9.

In accordance with yet another aspect of the invention there is provided Paragranulin: Thr Arg Cys Pro Asp Gly Gln Phe Cys Pro Val Ala Cys Cys Leu Asp

Pro Gly Gly Ala Ser Tyr Ser Cys Cys Arg Pro Leu Leu Asp, SEQ ID NO: 10.

In accordance with an embodiment of the invention there is provided a topical formulation comprising an effective amount of a Granulin of the invention in association with a pharmaceutically acceptable carrier for topical formulation.

In accordance with another aspect of the invention there is provided a method of healing wounds comprising applying to a wound site a Granulin of the invention.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 Figure 1A shows the HPLC chromatogram of a crude granule extract from inflammatory exudate cells, and B shows the chromatogram of a whole cell extract. The position of the granulins are marked by arrows. Note the absence in A of thymosin-β-4, a cytoplasmic marker peptide. The granule peptide markers HP-1 and HP-4 were identified as previously described (6), lysozyme was identified by amino terminal sequence analysis (unpublished). Thymosin-β-4 and its oxidation product were identified by Fast Atom Bombardment mass spectrometry;

FIG.2 Size-exclusion purification of granulin A, (panel A), granulin B, (panel B), and granulins C and D, (panel C). Size markers were substance P, CLIP and $ACTH_{1-39}$. Apparent m.wts for native granulin A, 2700; granulin B, 3200; granulin C, 1700; and granulin D, 3900;

FIG. 3 Purification of rat granulin from bone marrow; panel A shows the first HPLC chromatogram in acetonitrile/TFA, and B shows the second step of purification in

acetonitrile/HFBA. The bar in A corresponds to the region where the rat granulin elutes, and its elution position in B is marked with an arrow;

FIG. 4A Shows the sequence of the probe used to screen a human bone marrow cDNA library in gt 11;

FIG. 4B Shows a sequencing strategy;

FIG. 4C Shows the complete nucleotide sequence and deduced polypeptide sequence of granulin;

FIG. 5 Shows a comparison of the granulin-like domains;

FIG. 6 Is a Southern blot analysis of digested human DNA;

FIG. 7A Is a Northern blot analysis of granulin precursor mRNA;

FIG. 7B Is a Northern blot analysis of RNA from A431, A549 and SKMES-1; and

FIG. 7C Shows distribution of granulin mRNA.

MODES FOR CARRYING OUT THE INVENTION

MATERIALS AND METHODS

Tissue Sources

Blood was taken from healthy volunteers, prepared and fractionated using Ficoll-Hypaque (Pharmacia, Upsalla, Sweden) as previously described (6). Differential counts were obtained from the Hematology Department, Royal Victoria Hospital, Montreal, Canada. For structural studies, the first wash peritoneal exudate from patients with peritonitis was used as a source of leukocytes. Typically, this comprises from 75 to 95% neutrophils. Crude granule preparations were made by lysing the cells in Hank's buffered saline solution using a Cole Palmer Ultrasonic Homogeniser 4710 Series. Preparations were inspected visually under a microscope to ensure complete cell lysis. The cellular debris was pelleted

by centrifugation at 500 x g for 10 mins., and the supernatant inspected to ensure the complete removal of broken cells. The supernatant was then pelleted by centrifugation for 20 minutes at 5000 x g, and the crude granule pellet washed twice in HBSS. The two human granule peptides, HP-1 and HP-4, and the granule enzyme lysozyme, were used as granule markers, and the cytoplasmic peptide thymosin-β-4 was used as a marker for cytoplasmic contamination.

Extraction and Purification

Whole cell preparations or crude granule fractions were extracted by sonication using an acidic high-salt extraction medium as described elsewhere (15). The extract was then centrifuged at 2000 x g for 15 mins., and the pellets re-extracted. Pooled supernatants were then adsorbed on SepPak C_{18} cartridges (Waters Associates, Milford, Mass.) and eluted in 5 to 10 ml 80% acetonitrile in 0.1% TFA and the eluate lyophilized. The SepPak eluate was fractionated by reversed phase HPLC using a Waters C_{18}µBondapak column (7.8mm x 30cm) eluted over a three hour period using a gradient of 0 to 80% acetonitrile in 0.1% TFA throughout at an elution rate of 1.5ml min^{-1}. Aliquots of the eluted fractions were then screened by amino acid analysis. The fractions of interest were further purified by size-exclusion HPLC using two I-125 ProteinPak columns (Waters) connected in series, eluted isocratically in 40% acetonitrile in 0.1% TFA at 1 ml min^{-1} (16). Partially purified peptides were then purified to homogeneity using a second C-18µBondapak HPLC column (3.9mm x 30cm), with a gradient of 10 to 40% acetonitrile in 0.1% TFA throughout at 1.5 ml min^{-1} for 90 mins. The rat peptide was purified from the aspirated femural bone marrow of 50 Sprague-Dawley rats (Charles Rivers, St. Constant, Quebec), and extracted directly as outlined

above. SepPak eluates were fractionated on a C_{18}μBondapak column using a gradient of 4 to 48% acetonitrile in 0.1% TFA throughout over 1 hour at 1.5 ml min^{-1}. Fractions were screened by amino acid analysis, and granulin-like material further purified using the same column with a gradient of 20 to 40% acetonitrile in 0.13% HFBA throughout over one hour (15). Final purification was by size-exclusion HPLC as described above.

Amino Acid Analysis and Microsequencing

For amino acid analysis aliquots of the peptide were lyophilized in borosilicate glass tubes and hydrolyzed in an evacuated reacti-vial for 16 hours at 105°C with 6N HCl. Amino acid analysis was performed using a model 6300A Analyser (Beckman Instruments, Palo Alto, CA.). For microsequence analysis purified peptides were reduced with 10 mM dithiothreitol, or 2- mercaptoethanol, in 8M guanidine-HCl, 1 mM EDTA, 0.25M Tris, pH 8.5 for 1.5 hours at 37°C and then pyridylethylated with 2 μl 4-vinylpyridine (Aldrich Chemicals) under the same conditions.

The S-pyridylethylated peptides were then purified using a gradient of 5% to 60% acetonitrile containing 0.1% TFA throughout over 60 minutes with an initial 40 minute isocratic stage at 5% acetonitrile to elute polymeric vinylpyridine side products. The derivatized peptides were then submitted directly to sequence analysis or further processed by enzymatic digestion. The amino acid sequence determinations were carried out with an Applied Biosystem gas-phase sequenator (model 470A) as described in (17) but using a sequence program adapted from Speicher (18). The resulting phenylthiohydantoin (PTH)-amino acids were analysed by reverse-phase HPLC on the on line PTH-analyser (Applied Biosystem model 120A) and/or a stand

alone Varian HPLC unit as described previously (17). The PTH-yields for each standard were normalized according to a PTH-NorLeucine internal standard while the initial and repetitive yields were obtained by linear regression from the yields of selected stable PTH-derivatives. Sequence analysis of rat granulin and its fragments was undertaken at the McGill Peptide and Protein Sequencing facility located in the laboratory of Dr. Michael van der Rest at the Shriner's Hospital for Crippled Children in Montreal.

Enzymatic Digestion

S-pyridylethylated peptides were digested using trypsin (TPCK-treated, Sigma) chymotrypsin (Sigma), and S. aureus V8 protease (Sigma), at enzyme to substrate ratios of approximately 1 to 50 by weight. Digestions were performed at 37°C in 100 µl 50mM ammonium bicarbonate buffer, pH 8.3 for 3 hours, and terminated by the addition of 1 ml of 0.1% TFA. The proteolytic fragments were then fractionated by rp-HPLC on a C_{-18}µBondapak column using a gradient of 0 to 40% acetonitrile in 0.1% TFA throughout over 60 mins. at 1.5ml min^{-1}. Fractions were collected, aliquots removed for amino acid analysis, and then stored frozen at -80°C.

RESULTS

The HPLC profile of a typical extract of human inflammatory cells is shown in Figure 1. In addition to HP-1 and HP-4, several low abundance components are present. On the basis of amino acid composition analysis of these components three low abundance components that had unusually high levels of cystine were identified. These are labelled A, B and C/D, and were present in both whole cell extracts (FIG. 1B) and crude granule preparations (FIG. 1A). Each of these extracts was then further purified using size-exclusion HPLC, revealing that the component C/D

contained two peptides, one of which, D, eluted as a larger molecule than the other three. Each peptide was further purified on rp-HPLC. Their amino acid compositions are given in Table 1. The purified peptides were S-pyridylethylated, and amino terminal sequence analyses were performed, revealing that the four peptides were distinct but related molecules with no homology to any known protein. Because these peptides were associated with the granule fraction, we call them granulins A, B, C and D. Granulin D was run on reducing and non-reducing SDS-PAGE, and ran as a smaller molecule after reduction, indicating that it is probably a dimer. Only one peptide was recovered after S-pyridylethylation of granulin D suggesting that it is a homodimer. However, until a full sequence is determined the possibility that it is a heterodimer of closely related subunits can not be excluded. The rat defensin/corticostatins elute in the same region of the chromatogram as the granulins (19). Rat granulin (marked with a bar in Fig. 3a) was purified to apparent homogeneity by a further rp-HPLC step using HFBA as the counterion (Fig. 3b) and subjected to structural analysis essentially as described for the human peptides.

Granulin A is the most abundant of the human granulins, and was subject to a more detailed analysis. S-pyridylethylated peptide was digested with trypsin, chymotrypsin or S. aureus V8 protease. The fragments were isolated by HPLC, one fifth aliquots analysed by amino acid analysis, and appropriate fragments were then submitted to gas phase Edman microsequencing. Final recovery of the digestion products was between 150 and 300 picomoles. The overlap of the granulin A fragments is described in the legend to Table 2 together with similar data for the rat granulin. The two sequences are highly

conserved, as would be expected for regulatory molecules. Inflammatory exudates and bone marrow preparations are mixtures of cells, the exudates containing typically 70 to 95% granulocytes. When leukocytes from the blood of healthy donors was fractionated by density gradient centrifugation, granulins could be detected in the granulocyte pellet, but not in the interface where the mononuclear cells partition (data not shown).

The isolation and characterization of a novel family of leukocyte associated cystine-rich peptides, which are called granulins has been described. The sequence of one human granulin, A, and identification of three other human granulins, B, C and D has been set out and a fifth granulin isolated from rat bone marrow is partially sequenced. The most striking feature of their primary sequence is the high content of oxidised cysteine, over 20%, suggesting that the secondary structure of the granulins are an essential determinant of their biological activity. This is supported by evidence of rigid evolutionary constraint on their structures; the rat partial sequence is almost identical with human granulin A.

When the sequences were entered into the National Biomedical Research Foundation PIR data bank no homologies were found with other proteins, indicating that the granulins are a novel polypeptide family. Subsequently, however, two amino terminal sequences were published, epithelin 1 and 2, isolated from the rat kidney, which are homologous with the granulins. The kidney peptides are putative cytokines that have growth inhibitory and stimulatory properties on some epithelial cells <u>in vitro</u> (20). Rat granulin and the reported amino terminal sequence of epithelin 1 differ at only one residue; epithelin lacks the amino terminal glutamyl residue of rat granulin A. At

present it is not certain if both peptides are the product of the same, or different genes. It is also too early to determine whether the epithelins are intrinsic to renal cells, or if they are derived from blood borne cells trapped in the kidney. It is clear, however, that the two peptides from the kidney are members of a larger family, the granulins, and that a major source of the granulins, and probably also the epithelins, is from circulating leukocytes.

Rat granulin was isolated from bone marrow, indicating that granulins are of myeloid origin. Whether granulins are also synthesized in circulating leukocytes remains to be determined. Granulins were extracted from granulocyte rich preparations, and are recoverable from the granulocyte pellet after Ficoll-Hypaque density gradient centrifugation. This suggests that their cellular origin may be the neutrophil, however, it is possible that other granulocytes such as eosinophils, or contaminating monocytes, contribute to the granulin content of these extracts. Extracts of human platelets contained no detectable granulins (data not shown). It is possible that each granulin belongs to a distinct cell type, or that sub-classes of the same cell-type contain different granulins. These are issues best answered using immunolocalization procedures. The granulins co-purify in a crude granule extract. Granulocytes have several different granule subclasses, including a true secretory compartment (21) that can be activated independently of phagocytosis. The availability of suitable immunoassay techniques will permit the unambiguous location of the granulins to a cell type, and a subcellular compartment.

The granulins are a novel family of cystine-rich immunoinflammatory peptides. Their presence in circulating leukocytes and inflammatory exudates, and

their structural similarity with the rat epithelins (20), indicates their use in inflammation, wound repair and tissue remodeling.

Thus the isolation and characterization of a novel class of leukocyte peptides with possible cytokine-like activities which are called granulins is described. They are cystine-rich with molecular weights of approximately 6Kda, except for granulin D, which appears to be a dimer. The sequence of one member of this family, a 56 residue peptide, granulin A, and amino-terminal sequences for three other granulins from human peripheral leukocytes are described. A fifth related peptide was isolated and partially sequenced from rat bone marrow, suggesting that at least some of the granulin in peripheral leukocytes is preformed in the marrow. Rat granulin, and human granulin A, are closely related, showing that the granulin structures are highly conserved between species.

It has now been found that the precursor for the human granulins is a 593 residue glycoprotein, containing seven repeats of the 12-cysteine granulin domain. Gene expression is seen in the kidney, leukemic cell lines and fibroblasts, and very strongly in transformed epithelial cell lines, which both express the gene, and respond to the mature polypeptide.

Human leukocytes contain four granulin homologs, designated A, B, C and D. Their cellular distribution, and whether they are products of one or several genes under co-ordinate or independent control was unknown. To address these, and related issues, two oligonucleotide primers for PCR were synthesized corresponding to the amino terminal and mid-portion regions of granulin A (grnA). GrnA was chosen because it is the most abundant granulin in human leukocyte

extracts, and the complete sequence of A, but only partial sequences of the other granulins, was available (22,23). Two PCR amplification products were obtained from human genomic DNA. One was unrelated to the granulins. The other fragment which represented part of the GrnA gene split by a 79 nucleotide intron (Fig. 4A) was ^{32}P labelled and used to probe a human bone marrow cDNA library (Clontech). Positive clones were analyzed and the nucleotide sequence of the grnA precursor was then obtained as outlined in Fig. 4B.

The sequence (Fig. 4C) predicts a protein of 593 residues, with a probable signal peptide (24) extending to residue 17. It contains the 56 residue grnA sequence, as expected, and also six other 12-cysteine granulin-like domains, including B, C and D, hitherto known only from N-terminal sequences. The precursor contains two novel Cys-12 domains, E and F, and a degenerate granulin domain, G, with 10 cysteines. The positions of the cysteines are highly conserved (Fig. 5) as are certain other residues such as Asp_{39}, His_{42} and Pro_{48}. An eighth domain at the N-terminus, (paragranulin, Fig. 5) contains only 6 cysteines corresponding to the amino-terminal half of a granulin domain.

There are 5 potential N-glycosylation sites (Fig. 4C), including one at Asn_5 of grnC. GnrC hydrolysates contain amino sugars and microsequencing gave a blank at residue 5 (N=2), consistent with glycosylation. The inter-domain sequences show little homology, except for a pro-ala dipeptide, found midway between each domain except F and G. Mono- or dibasic sequences, which are frequent sites of proteolysis in peptide processing (25), flank granulin domains C, D and E, but not A or B, both of which have been isolated as excised peptides. Thus nothing can be

reliably inferred about post-translational cleavage mechanisms of the granulin precursor. There are no obvious trans-membrane sequences beyond the signal peptide (26).

Southern blot analysis of human genomic DNA suggests that only one gene hybridizes with the granulin probe (Fig. 6). Significant interspecies sequence conservation exists, since DNA blots (Clontech) from human, monkey, rat, mouse, dog, bovine and rabbit, but not chicken, or yeast, hybridized with the granulin probe (data not shown). The blots probably underestimate species distribution since granulin-like peptides have been isolated from teleost hematopoietic tissue (H.P.J. Bennett, personal communication).

Four myelogenous leukemic cell lines of diverse linage, HL-60, U937, K562, KMOE, each expressed a granulin mRNA of approximately 2.3 Kb, as did murine 3T3 fibroblasts, but by far the strongest hybridization was detected in epithelial cell lines (Fig. 7A). These cells produce two granulin mRNA species, at 2.3 and 2.5 Kb (Fig. 7B), the origin and significance of which is under investigation. The abundant expression of granulin mRNA in epithelial cell lines is particularly interesting, since epithelial cells, including A431 (23), respond to, and presumably have receptors for, epithelin 1 (and grnA). The majority of solid tumors are epithelial in origin, thus the propensity of transformed epithelial cells to express granulin mRNA, and respond to the mature peptide, may have important pathophysiological consequences. In tissues, very strong hybridization was obtained with rabbit kidney RNA (Fig. 7C). This resembles EGF gene expression, which is a hundred times higher in the kidney than any other organ, except the submaxillary gland in rodents (27).

The widespread expression of the granulin gene in cells of diverse lineage; the effects of some of the granulins/epithelins on cell proliferation (23), and the high degree of sequence conservation noted earlier (22), and corroborated here by interspecies DNA hybridization, cumulatively support a fundamental regulatory role for granulins/epithelins. The presence of all four known granulins, plus three novel granulin-like sequences in a common precursor, was highly unexpected. The iterative nature of pro-granulin draws unavoidable comparisons with the EGF precursor (28,29). Both the pro-granulin and EFT-precursor (27) mRNAs are very abundant in the kidney. Biologically, EGF and granulin/epithelins have similar actions on epithelial cells (23), but probably different receptors (23). Whether the similarities are significant, or merely coincidental, is unclear. Unlike the EGF-precursor, pro-granulin has no transmembrane segment or cytoplasmic domain (28,29) implying different post-translational pathways for the two proteins. Multiple sequential repeats of cysteine-rich domains is a recurrent structural motif among regulatory proteins (28,29,30,31), wherein typically, one exon delineates one cysteine-rich domain (30,32,33). In contrast, the PCR amplified fragment of grnA is bisected by an intron (Fig. 4A), and preliminary results show that introns bisect domains G, F, B, C and D.

In each case the cysteines align with an approximately mirror-image symmetry around the bisecting intron. Genomically, therefore, pro-granulin bears little resemblance to the EGF-precursor.

Proerythroid leukemic cells express granulin mRNA (Fig. 7A), but seem unlikely to be involved in regulating epithelial proliferation. Similarly, the

strong expression of granulin in RNA in kidney, but not other tissues, may imply functions unrelated to epithelial cell mitogenesis. Thus granulins may have multiple biological activities; indeed distinct activities may be associated with different domains, as already proposed for epithelins 1 and 2 (23). The availability of cloned granulins permit these questions to be addressed.

Fig. 4. (A) shows the sequence of the probe used to screen a human bone marrow cDNA library in gt11 (Clontech, Palo Alto, CA.). The probe was generated by PCR of human DNA, with forward and reverse primers (underlined) based on the amino acid sequence of granulin A. The nucleotides in lower case represent an intron in the coding region of granulin A. Fig. 4B shows the sequencing strategy. The nucleotide sequence is a composite of three sequences HBM12, HBM3 and HNM4. Restriction sites for EcoRI, KpnI and SacI are shown; sequence was obtained using M13 or T3 primers (f,g,l,n,o) or custom-synthesized 17-mer sequence specific primers (a,b,c,d,e,h,i,j,k,m,p). Arrows indicate the direction of sequencing and overlap. Fig. 4C shows the complete nucleotide sequence and deduced polypeptide sequence of granulin. The nucleotides are numbered from the initiator codon (ATG), and the amino acids from the probable signal peptide cleavage site. Underlined sequences correspond to sequences previously determined by gas-phase microsequencing of purified granulins. Possibly N-glycosylation sites are indicated by an asterisk (*), and the stop codon is shown by a #. The boxed nucleotides corresponds to a polyadenylation signal. Clone HBM12 starts at nucleotide 6. The sequences of clones HBM3 and HBM4 differ in the 5'-untranslated region as indicated by the bifurcation in the nucleotide sequence.

METHODS. To generate the grnA probe, degenerate oligonucleotide primers (forward primer, 5'-CGATGTGAAGTG(T/C)GA(T/C)ATGGA-3'; reverse primer, 5'-CTGGCATGTGGTT(T/C)TC(A/G)CA(G/A)CA-3') were synthesized (Sheldon Biotechnology Centre, McGill University) and used in the polymerase chain reaction (PCR) with 2µg of genomic DNA as template (34). The amplified product was subcloned into the plasmid vector Bluescript KSII+ and its identity confirmed by double stranded dideoxynucleotide sequencing with Sequenase (USB). This fragment was labeled with ^{32}P by nick-translation (Boehringer Mannheim) and used to probe a human bone marrow cDNA library in λgt11 (Clontech, Palo Alto, CA.) consisting of 1.51 $X10^6$ independent clones. Duplicate nitrocellulose filters (Schleicher and Schuell) were prehybridized in 5X SSC, 5X Denhardt's reagent, 0.2% SDS at 37°C for 5 hours. Hybridization was in 5X SSC, 2.5X Denhardt's reagent, 0.2% SDS, 50% formamide, 10% polyethylene glycol with $1x10^7$ cpm probe at 37°C for 12 hours. Filters were washed twice for 45 minutes in 2X SSC, 0.1% SDS at 58°C and exposed at -70°C with Kodak X-Omat film with an intensifying screen. 16 positives were obtain from $3x10^5$ clones screened. The cDNA insert from clone HMB12 was digested with KpnI and SacI, and the resulting cDNA fragments subcloned into Bluescript KSII+. Nucleotide sequence was determined by double stranded dideoxy sequencing using Sequenase (USB). The sequence of HBM12 lacked an initiator methionine. The remaining positive clones were analysed using PCR with λgt11 sequence specific primers in combination with a primer, c112rp, corresponding to nucleotides 45 to 61 of clone HBM12. Only two clones, HBM3 and HBM4 contained inserts longer than HBM12. Their 5' sequence was obtained by dideoxy sequencing of single stranded templates generated by asymmetric PCR (35)

using a forward primer specific for λgt11 and primer c112rp as the reverse primer. Only the 5'-ends of HBM3 and HBM4 were sequenced. The probable identity with HBM12 was established by PCR mapping using primer pairs b,d; e,k; i,k; h,grnAr; grnBf,grnAr; b,grnFr. grnAr, grnBf and grnFr are primers specific for granulins A, B and F and were not used for sequencing.

Fig.5. Comparison of the granulin-like domains. Residues occuring three or more times are boxed, and dashes have been introduced to align the cysteines. The domain boundaries were determined by gas-phase microsequencing and by comparison with the sequence of grnA. The domains are located at amino acids, 264-319, grnA; 169-244, grnB; 347-400, 425-479, grnD; 501-506, grnE; 106-162, grnF; 41 grnG and 1-27, paragranulin. The order of domains is: paragranulin-G-F-B-A-C-D-E. They are not alphabetically aligned because several domains were isolated and named as discrete proteins before their common origin was known.

Fig. 6. Southern blot analysis of human DNA, digester with BglII, PstI, BamHI, HindIII, and EcoRI, 5ug per lane (Clontech, Palo Alto, CA.), probed with a ^{32}P nick-translated 1890 bp EcoRi/SacI fragment from clone HBM12. Hybridization was in 0.5M NaH_2PO_4, pH 7.2, 7% SDS, 1mM EDTA at 65°C for 20 hours. The blot was washed twice at 65°C, 30 minutes each, in 40 mM NaH_2PO_4, pH 7.2, 5% SDS, 1mM EDTA and 40mM NaH_2PO_4, pH 7.2, 1% SDS, 1mM EDTA. The autoradiogram was obtained by exposing the blot to Kodak X-Omat film with intensifying screen at -70°C for 5 hours.

Fig. 7. Northern blot analysis of: A. granulin precursor mRNA in the following cell lines, HL-60 (promycelocytic leukemia), U937 (histocytic leukemia), K562 and KMOE (proerythroid leukemias), A431 (epidermoid carcinoma), A549 (lung epithelial

carcinoma), SKMES-1 (lung squamous carinoma), CHO-KI (chinese hamster ovaries), and murine Balb/C 3T3 fibroblasts, exposed for 10 days. (B). RNA from A431, A549 and SKMES-1, exposed for 20 hours. (C). Distribution of granulin mRNA in rabbit tissues; heart, liver, spleen, kidney, bone marrow, exposed for 4 days.

METHODS. Total cellular RNA was isolated from cell lines and rabbit tissues by the acid guanidinium thiocyanate-phenol-chloroform extraction method (15), 30µg (cell lines) or 50µg (rabbit tissues) RNA was denatured with glyoxal, electrophoresed on a 1.1% agarose gel in 10 mM NaH_2PO_4, pH 6.8, transferred to nylon membranes (ZetaProbe, BioRad) by capillary blotting with 10mM NaOH and fixed by baking at 80°C for 2 hours. The membranes were hybridized at 65°C for RNA extracted from cell lines or 60°C for rabbit tissue RNA in 0.5M NaH_2PO_4, pH 6.8, 7% SDS, 1mM EDTA for 24 hours with the same probe described in Figure 6. The membranes were washed at hybridization temperature for 30 mins. in 40 mM NaH_2PO_4, pH 6.8, 5% SDS, 1mM EDTA and 30 minutes in 40mM NaH_2PO_4, pH 6.8, 1% SDS, 1mM EDTA. Autoradiograms were obtained by exposing the blots to Kodak X-Omat film with intensifying screens at -70°C.

REFERENCES

1. Selsted M.E., Brown D.M., Delange R.J., and Lehrer R.I. (1983) J. Biol. Chem. 258, 14485-14489.
2. Zhu Q., Hu J., Mulay S., Esch F., Shimasaki S., and Solomon S. (1988) Proc. Natl. Acad. Sci. (USA) 85, 592-596.
3. Mars W.M., van Tuinen P., Drabkin H., White J., and Saunders G. (1988) Blood 71, 1713-1719.
4. Ouellette A.J., Greco R.M., James M., Frederick, D., Naftilan J., and Fallon J.T. (1989) J. Cell

Biol., 108, 1687-1695.

5. Ganz T., Selsted M.E., Szklarek, D., Harwig S.S.l., Daher K., and Lehrer R.I. (1985), J. Clin. Invest. 76, 1427-1435.

6. Singh A., Bateman A., Zhu Q., Shimasaki S., Esch F., and Solomon S. (1988) Biochem. Biophys. Res. Comm. 155, 524-529.

7. Zhu Q., Bateman A., Singh A., Solomon S. (1989) Endocrine Research 15, 129-149.

8. MacLeod R. J. Hamilton J.R., Bateman A., Belcourt D., Hu J., Bennett H.P.J., and Solomon S. (1991) Proc. Natl. Acad. Sci. (USA), In Press.

9. Territo M.C., Ganz T., Selsted M.E., and Lehrer R.I., (1989) J. Clin. Invest. 84, 2017-2020.

10. Laerum O.D., and Paukovits W.R., (1988) in The Inhibitors of Hematopoiesis, eds Najman A. Guignon M., Gorin N-C., and Mary J-Y., (John Libbey Eurotext, London, Paris) 21-30.

11. White M.V., and Kaliner M.A. (1987) 139, 1624-1630.

12. Doherty D.E., Downey G.P., Worthern G.S., Haslett C., and Henson P.M. (1988) Lab. Invest 59, 200-213.

13. Willemze R., Walker R.I., Herion J.C., and Palmer J.G. (1978), Blood 51, 21-28.

14. Benestad H.B., Hersleth I.B. (1984), Blut 48, 201-211.

15. Bennett H.P.J., Browne C.A., Solomon S. (1981) Biochem. 20, 4530-4538.

16. Bennett H.P.J., Browne C.A., and Solomon S. (1983) Anal Biochem. 128 121-129.

17. Lazure C. Saayman H.S., Naudé R.J., Oelofsen W., and Chrétien M., (1989) Int. J. Peptide Res. 33, 46-58.

18. Speicher D.W. (1989) in Techniques in Protein Chemistry (Hugli T., ed), Academic Press,

San Diego, pp 24-35.

19. Belcourt D., Bateman A., Singh A., Lazure C., Bennett H.P.J., and Solomon S. (1990) 72nd Annual Meeting of the Endocrine Society, Atlanta, Georgia, pp. 276 (abs.).

20. Shoyab M., McDonald V.L., Byles C., Todaro G., and Plowman G.D. (1990) Proc. Natl. Acad. Sci. (USA) 87, 7912-7916.

21. Dewalt B., Bretz U., and Baggiolini M., J. Clin. Invest. (1982) 36, 518-525.

22. Bateman, A., Belcourt, D., Bennett, H.P.J. Lazure, C., and Solomon, S. Biochem. Biophys. Res. Comm., 173, 1161-1168, (1990).

23. Shoyab, M., McDonald, V.L. Byles, C., Todaro, G., and Plowman G.D., Proc. Natl. Acad. Sci. (USA), 87, 7912-7916, (1990).

24. von Heijne, G., J. Mol. Biol., 184, 99-105 (1985).

25. Lazure, V., Seidah N.G., Pelaprat, D., and Chretien, M., Can. J. Biochem. Cell Biol., 61, 501-515, (1983).

26. Kyte, R.J., and Doolittle, R.F., J. Mol. Biol., 157, 105-132, (1982).

27. Rall, L.B., Scott J., Bell G.I., Crawford, R.J. Penschow, J.D., Niall, H.D., and Coghlan J.P., Nature, 313, 228-231, (1985).

28. Gray, A., Dull, T.J., and Ullrich, A., Nature, 303, 722-725, (1983).

29. Scott, J., Urdea, M., Quiroga M. Sanchez-Pescador R., Fong, N., Selby, M., Rutter, W., and Bell G., Science, 221, 236-240 (1983).

30. Shimasaki, S., Koga, M., Esch, F., Cooksey, K., Mercado, M., Koba A., Ueno N., Ying, S-Y., Ling, N., and Guillemin, R., Proc. Natl. Acad. Sci. (US), 85, 4218-4222, (1988).

31. Bevilacqua, M. P., Stengelin, S., Gimbrone, M.A.,

and Seed, B., Science 243, 1160-1165, (1989).

32. Bell, G.I., Fong, N.M., Stempien N.M., Wormsted, M.A., Caput, D., Ku, L., Urdea, M.S., Rall, L.B., and Sanchez-Pescador, R., Nucleic Acids Research, 14, 8427-8446, (1986).

33. Collins, T., Williams A., Johnston, G.I., Kim, J., Eddy, R., Shows, T., Gimbrone, M.A. Jr., Bevilacqua, M.P., J. Biol. Chem., 266, 2466-2473, (1991).

34. Saiki, R.K. Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., Erlich, H.A., Science, 239, 487-491, (1988).

35. Gyllensten, U.B. and Erlich, H.A., Proc. Natl. Acad. Sci. (USA), 85, 7652-7656 (1988).

36. Chomczynski, P. and Sacchi, N., Anal., Biochem., 162, 156-159, (1987).

Following the procedures described herein before carp Granulin: VIHCDAATICPDGTICCLSPYGMBGQCCRDGIHCCRHGYHCDSRTTHCL was isolated and characterized. It was found to be homologous with rat and human granulins but not identical.

The carp Granulin causes an increase in ^{3}H-thymidine incorporation in a keratinocyte cell line which indicates increased cell growth in keratinocytes and thus wound healing.

The above described Granulin A causes a fall in ^{3}H-thymidine incorporation in A-431 cells, a human epidermal carcinoma cell line. The human granulins of the invention retard growth of A-431 cells.

Pharmaceutical Compositions

The granulins of the invention are suitably employed as the active ingredient in pharmaceutical compositions for topical application.

Suitably the granulins may be incorporated in a topical formulation in an amount of 5 to 50µg/ml of

ointment and are applied to a wound site in an amount of 5 to 50 $\mu g/cm^2$ of wound site. A preferred treatment amount of the granulins is about 10$\mu g/cm^2$ of wound site suitably applied three times a day.

The topical pharmaceutical composition may be in the form of a cream, ointment, gel, lotion or other formulation suitable for application to the skin, and thus comprise a granulin of the invention admixed with an acceptable carrier for topical application.

TABLE 1: The amino acid compositions of purified granulins. The recovery of cysteine in this system is variable and is between 65 to 80%. Values have not been corrected for background contamination or oxidation. Tryptophan was not determine. Predicted values for granulin A from the gas-phase sequence determinations are given in brackets.

Amino Acid	Granulin A	Granulin B	Granulin C	Granulin D	Rat Granulin
ASX	5.7 (5)	6.5	5.2	5.4	4.0
THR	4.9 (5)	2.5	2.6	2.9	5.1
SER	2.3 (2)	5.1	6.2	4.6	1.2
GLU	7.4 (6)	3.4	8.1	7.3	5.6
PRO	3.2 (3)	5.6	5.0	4.5	2.4
GLY	5.7 (5)	3.7	4.8	6.2	5.5
ALA	2.8 (3)	2.0	1.9	4.5	2.7
CYS	7.0(12)	7.2	7.2	9.1	8.2
VAL	3.0 (3)	1.9	2.9	2.2	2.2
MET	0.7 (1)	1.5	0	0	0
ILE	0.95(1)	1.0	0.85	0.8	0.7
LEU	1.1 (1)	3.7	1.0	2.6	3.2
TYR	0.9 (1)	1.0	1.1	1.0	1.1
PHE	2.0 (2)	0	0	0	1.7
HIS	2.5 (2)	1.7	2.8	3.6	2.3
LYS	2.0 (2)	2.3	0	1.8	1.9
ARG	1.3 (1)	2.3	1.0	3.0	1.7

TABLE 2: Structural analysis of five members of the granulin family. The proposed structure for granulin A was determined by overlapping two amino terminal sequences, (1-11), and (1-23); tryptic fragments (4-18), (19-52), and V8 protease fragment (37-56), and chymotryptic fragment (47-56). The proposed structure of rat granulin is based partly upon the direct sequencing of the peptide itself (i.e., the sequence 1 through 21) and its proteolytic fragments (i.e., fragments corresponding to 4 to 18, 19 to 31, 32 to 46, and 32 to 51 sequences). Alignment of the fragments is based upon the clear homology of rat granulin with human granulin A. The sequence of residues 26 to 31 (in parenthesis) could not be determined and the sequence shown is based upon the amino acid composition of fragment 19 to 31. This fragment is a major tryptic clevage product. No evidence of arginine was found, however, it is possible that the fragment contains a lysine residue which co-elutes with pyridylethyl-cysteine in the amino acid analysis system. B signifies either aspartic acid or asparagine, and X signifies an unassigned residue.

Granulin A:	DVKCDMEVSCPDGYTCCRLQSGAWGCCPFTQAVCCEDH-IHCCPAGFTCDTQKGTCE.
Rat Granulin:	EVKCDLEVSCPDGYTCCRLNTGAWG(CCPFSB)AVCCE-DHIHCCPAGFTCXTQ.
Granulin B:	VMCPDARSRCPDGHTCCELPSGKYGCCPMPNATCCSDH-LHCCPQDTVCDLIQSKCI.
Granulin C:	VPCDXVSSCPSSDTCCQLTSGEHGCCPIPEAVC.
Granulin D:	IGCDQXDTSSCCPDG.

Abbreviations.
TFA, trifluoroacetic acid: HFBA, heptafluorobutyric acid; rp-HPLC reverses phase high performance liquid chromatography; PTH, phenylthiohydantoin.

(I) GENERAL INFORMATION

(i) APPLICANT: SOLOMON, SAMUEL

(ii) TITLE OF INVENTION: GRANULINS FROM LEUKOCYTES

(iii) NUMBER OF SEQUENCES: Six (6)

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(E) COUNTRY: Canada

(F) ZIP: H3A 1A1

(v) COMPUTER READABLE FORM:

(A) Medium Type: Diskette 3.50 Inch 1.44 Mb

(B) COMPUTER: Olivetti M300-386 SX

(C) OPERATING SYSTEM: MS DOS

(D) SOFTWARE: ASCII Format (Word for Windows)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER - U.S. Patent
Application, Ser. No. Unknown

(B) FILING DATE: 3 Feb. 1992

(C) CLASSIFICATION: Unknown

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER - U.S. Patent
Application Ser. No. 07/627,490

(B) FILING DATE: 14 Dec. 1990

(C) CLASSIFICATION: 154

SEQ ID NO:1

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 55 AMINO ACIDS

(B) TYPE: AMINO ACID

(C) STRANDEDNESS: NOT APPLICABLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULAR TYPE: PEPTIDE

(vi) ORIGINAL SOURCE: HUMAN NEUTROPHILS

Asp Val Lys Cys Asp Met Glu Val Ser Cys Pro Asp Gly
5 10

Tyr Thr Cys Cys Arg Leu Gln Ser Gly Ala Trp Cys Cys
15 20 25

Pro Phe Thr Gln Ala Val Cys Cys Glu Asp His Ile His
30 35

Cys Cys Pro Ala Gly Phe Thr Cys Asp Thr Gln Lys Gly
40 45 50

Thr Cys Glu
55

SEQ ID NO: 2

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 56 AMINO ACIDS

(B) TYPE: AMINO ACID

(C) STRANDEDNESS: NOT APPLICABLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PEPTIDE

(vi) ORIGINAL SOURCE: HUMAN NEUTROPHILS

Val Met Cys Pro Asp Ala Arg Ser Arg Cys Pro Asp
5 10

Gly Ser Thr Cys Cys Glu Leu Pro Ser Gly Lys Tyr Gly
15 20 25

Cys Cys Pro Met Pro Asn Ala Thr Cys Cys Ser Asp His
30 35

Leu His Cys Cys Pro Gln Asp Thr Val Cys Asp Leu Ile
40 45 50

Gln Ser Lys Cys Leu
55

SEQ ID NO: 3

(i) SEQUENCE CHARACTERISTICS:

(A) SEQUENCE LENGTH: 54 AMINO ACIDS

(B) TYPE: AMINO ACID

(C) STRANDEDNESS: NOT APPLICABLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULAR TYPE: PEPTIDE

(iv) ORIGINAL SOURCE: HUMAN NEUTROPHILS

Val Pro Cys Asp Asn Val Ser Ser Cys Pro Ser
5 10

Ser Asp Thr Cys Cys Gln Leu Thr Ser Gly Glu Trp
15 20

Gly Cys Cys Pro Ile Pro Glu Ala Val Cys Cys Ser
25 30 35

Asp His Gln His Cys Cys Pro Gln Arg Tyr Thr Cys
40 45

Val Ala Glu Gly Gln Cys Gln
50

SEQ ID NO: 4

(i) SEQUENCE CHARACTERISTICS:

(A) SEQUENCE LENGTH: 55 AMINO ACIDS

(B) TYPE: AMINO ACID

(C) STRANDEDNESS: NOT APPLICABLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PEPTIDE

(vi) ORIGINAL SOURCE: HUMAN NEUTROPHILS

Ile Gly Cys Asp Gln His Thr Ser Cys Pro Val
5 10

Gly Gly Thr Cys Cys Pro Ser Gln Gly Gly Ser Trp
15 20

Ala Cys Cys Gln Leu Pro His Ala Val Cys Cys Glu
25 30 35

Asp Arg Gln His Cys Cys Pro Ala Gly Tyr Thr Cys
40 45

Asn Val Lys Ala Arg Ser Cys Glu
50 55

SEQ ID NO: 5

(i) SEQUENCE CHARACTERISTICS

(A) SEQUENCE LENGTH: 51 AMINO ACIDS

(B) TYPE: AMINO ACID

(C) STRANDEDNESS: NOT APPLICABLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULAR TYPE: PEPTIDE

(vi) ORIGINAL SOURCE: RAT BONE MARROW

Glu Val Lys Cys Asp Leu Glu Val Ser Cys Pro Asp
5 10

Gly Tyr Thr Cys Cys Arg Leu Asn Thr Gly Ala Trp
15 20

Gly Cys Cys Pro Phe Ser Asp Ala Val Cys Cys Glu
25 30 35

Asp His Ile His Cys Cys Pro Ala Gly Phe Thr Cys
40 45

--- Thr Gln
50

The exact identity of position 31 is not yet determined; it is either Asp or Asn. The identity of position 49 is not known.

SEQ ID NO: 6

(i) SEQUENCE CHARACTERISTICS:

(A) SEQUENCE LENGTH: 49 AMINO ACIDS

(B) TYPE: AMINO ACID

(C) STRANDEDNESS: NOT APPLICABLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULAR TYPE: PEPTIDE

(vi) ORIGINAL SOURCE: CARP SPLEEN

Val Iso His Cys Asp Ala Ala Thr Ile Cys Pro Asp
5 10

Gly Thr Ile Cys Cys Leu Ser Pro Tyr Gly Asp Met
15 20

Gly Gln Cys Cys Arg Asp Gly Ile His Cys Cys Arg
25 30 35

His Gly Tyr His Cys Asp Ser Arg Thr Thr His Cys
40 45

Leu

The exact identity of position 23 is not yet determine; it is either Asp or Asn.

SEQ ID NO: 7

(i) SEQUENCE CHARACTERISTICS:

(A) SEQUENCE LENGTH: 56 AMINO ACIDS

(B) TYPE: AMINO ACID

(C) STRANDEDNESS: NOT APPLICABLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULAR TYPE: PEPTIDE

(v) ORIGINAL SOURCE: HUMAN NEUTROPHILS

Asp Val Glu Vys Gly Glu Gly His Phe Cys His Asp Asp
5 10

Gln Thr Cys Cys Arg Asp Asn Arg Glu Gly Trp Ala Cys
15 20 25

Cys Pro Tyr Ala Gln Gly Val Cys Cys Ala Asp Arg Arg
30 35

His Cys Cys Pro Ala Gly Phe Arg Cys Ala Arg Arg Gly
40 45 50

Thr Lys Cys Leu
55

SEQ ID NO: 8

(i) SEQUENCE CHARACTERISTICS:

(A) SEQUENCE LENGTH: 57 AMINO ACIDS

(B) TYPE: AMINO ACID

(C) STRANDEDNESS: NOT APPLICABLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULAR TYPE: PEPTIDE

(vi) ORIGINAL SOURCE: HUMAN NEUTROPHILS

Ala Ile Gln Cys Pro Asp Ser Gln Phe Glu Cys Pro Asp
5 10

Phe Ser Thr Cys Cys Val Met Val Asp Gly Ser Trp Gly
15 20 25

Cys Cys Pro Met Pro Gln Ala Ser Cys Cys Glu Asp Arg
30 35

Val His Cys Cys Pro His Gly Ala Phe Cys Asp Leu Val
40 45 50

His Thr Arg Cys Ile
55

SEQ ID NO: 9

(i) SEQUENCE CHARACTERISTICS:

(A) SEQUENCE LENGTH: 57 AMINO ACIDS

(B) TYPE: AMINP ACID

(C) STRANDEDNESS: NOT APPLICABLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PEPTIDE

(vi) ORIGINAL SOURCE: HUMAN NEUTROPHILS

Gly Gly Pro Cys Gln Val Asp Ala His Cys Ser Ala Gly
5 10

His Ser Cys Ile Phe Thr Val Ser Gly Thr Ser Ser Cys
15 20 25

Cys Pro Phe Pro Glu Ala Cys Gly Asp Gly His His Cys
30 35

Cys Pro Arg Gly Phe His Cys Ser Ala Asp Gly Arg Ser
40 45 50

Cys Phe

SEQ ID NO: 10

(i) SEQUENCE CHARACTERISTICS:

(A) SEQUENCE LENGTH: 30 AMINO ACIDS

(B) TYPE: AMINO ACID

(C) STRANDEDNESS: NOT APPLICABLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULAR TYPE: PEPTIDE

(vi) ORIGINAL SOURCE: HUMAN NEUTROPHILS

Thr Arg Cys Pro Asp Gly Gln Phe Cys Pro Val Ala Cys
5 10

Cys Leu Asp Pro Gly Gly Ala Ser Tyr Ser Cys Cys Arg
15 20 25

Pro Leu Leu Asp
30

CLAIMS

1. Novel cystine-rich leukocyte granulins.

2. A granulin of claim 1, which is Granulin A: DVKCDMEVSCPDGYTCCRLQSGAWGCCPFTQAVCCEDHIHCCPAGFTCDTQKGT CE, SEQ ID NO: 1.

3. A granulin of claim 1, which is Granulin B: VMCPDARSRCPDGGTCCELPSGKYGCCPMPNATCCSDHLHCCPQDTVCDLIQSK CL, SEQ ID NO: 2.

4. A granulin of claim 1, which is Granulin C: VPCDXVSSCPSSDTCCQLTSGEHGCCPIPEAVC, SEQ ID NO: 3.

5. A granulin of claim 1, which is Granulin D: IGCDQXDTSSCCPDG, SEQ ID NO: 4.

6. A granulin of claim 1, which is Rat Granulin: EVKCDLEVSCPDGYTCCRLNTGAWG(CCPFSB)AVCCEDHIHCCPAGFTCXTQ, SEQ ID NO: 5.

7. A granulin of claim 1, which is Carp Granulin: VIHCDAATICPDGTICCLSPYGBMGQCCRDGIHCCRHGYHCDSRTTHCL, SEQ ID NO: 6.

8. A granulin of claim 1, which is Granulin E: Asp Val Glu Cys Gly Glu Gly His Phe Cys His Asp Asp Gln Thr Cys Cys Arg Asp Asn Arg Glu Gly Trp Ala Cys Cys Pro Tyr Ala Gln Gly Val Cys Cys Ala Asp Arg Arg His Cys Cys Pro Ala Gly Phe Arg Cys Ala Arg Arg Gly Thr Lys Cys Leu, SEQ ID NO: 7.

9. A granulin of claim 1, which is Granulin F: Ala Ile Gln Cys Pro Asp Ser Gln Phe Glu Cys Pro Asp Phe Ser Thr Cys Cys Val Met Val Asp Gly Ser Trp Gly Cys Cys Pro Met Pro Gln Ala Ser Cys Cys Glu Asp Arg

Val His Cys Cys Pro His Gly Ala Phe Cys Asp Leu Val His Thr Arg Cys Ile, SEQ ID NO: 8.

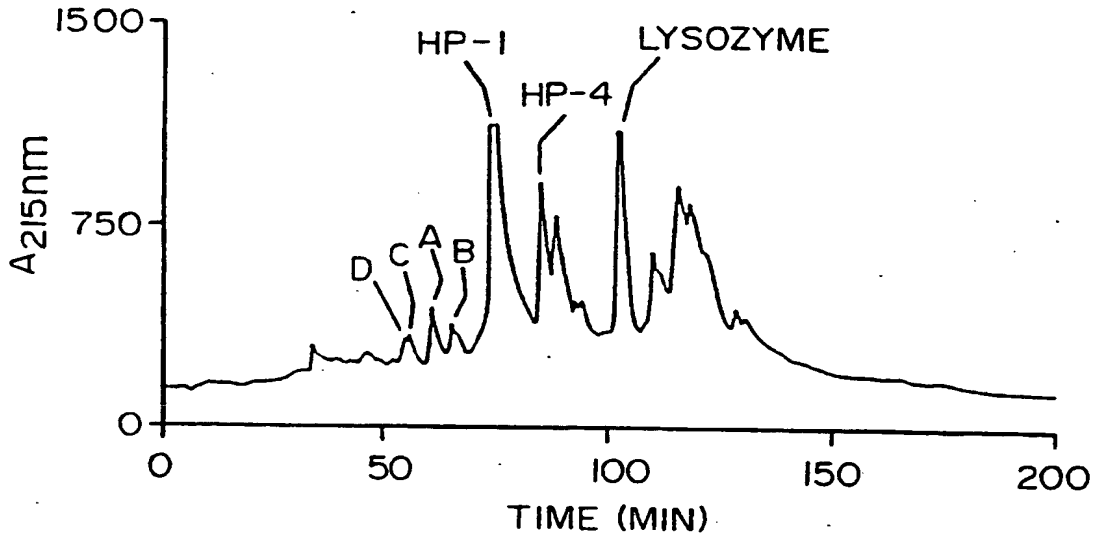
10. A granulin of claim 1, which is Granulin G: Gly Gly Pro Cys Gln Val Asp Ala His Cys Ser Ala Gly His Ser Cys Ile Phe Thr Val Ser Gly Thr Ser Ser Cys Cys Pro Phe Pro Glu Ala Cys Gly Asp Gly His His Cys Cys Pro Arg Gly Phe His Cys Ser Ala Asp Gly Arg Ser Cys Phe, SEQ ID NO: 9.

11. A granulin of claim 1, which is Paragranulin: Thr Arg Cys Pro Asp Gly Gln Phe Cys Pro Val Ala Cys Cys Leu Asp Pro Gly Gly Ala Ser Tyr Ser Cys Cys Arg Pro Leu Leu Asp, SEQ ID NO: 10.

12. A topical formulation containing a granulin of claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11, in association with a pharmaceutically acceptable carrier for topical application.

13. A topical formulation comprising an effective keratinocytic amount of the granulin of claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11, in association with a pharmaceutically acceptable carrier for topical application.

14. A granulin of claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11, for use in healing a wound site.


1500
750
0
A215nm
HP-1
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D
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50
100
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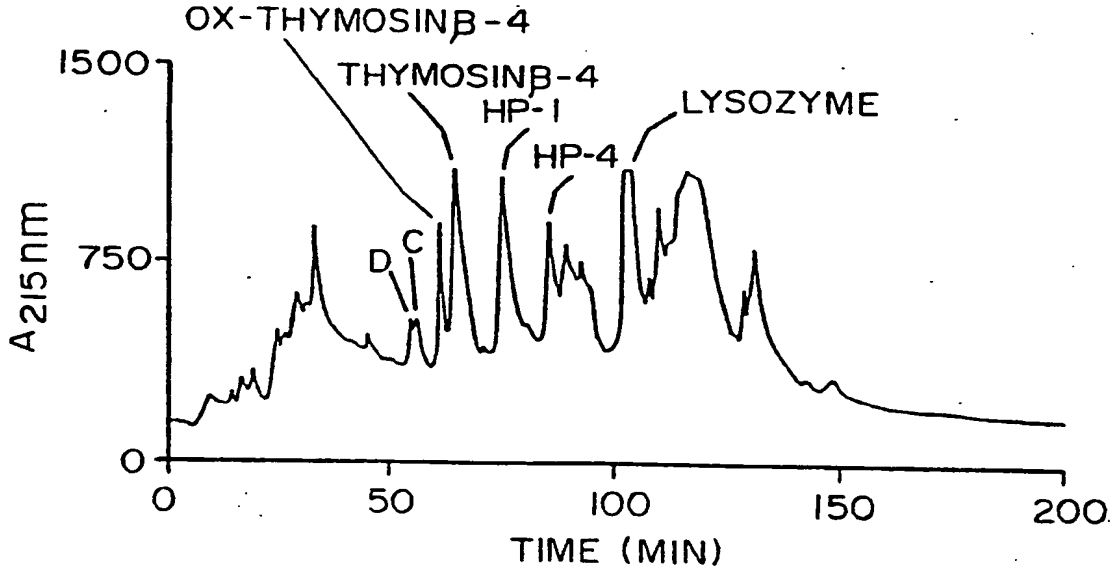
200
TIME (MIN)

Fig. 1A


OX-THYMOSINβ-4
THYMOSINβ-4
HP-1
LYSOZYME
HP-4
1500
750
0
A215nm
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150
200
TIME (MIN)

Fig. 1B

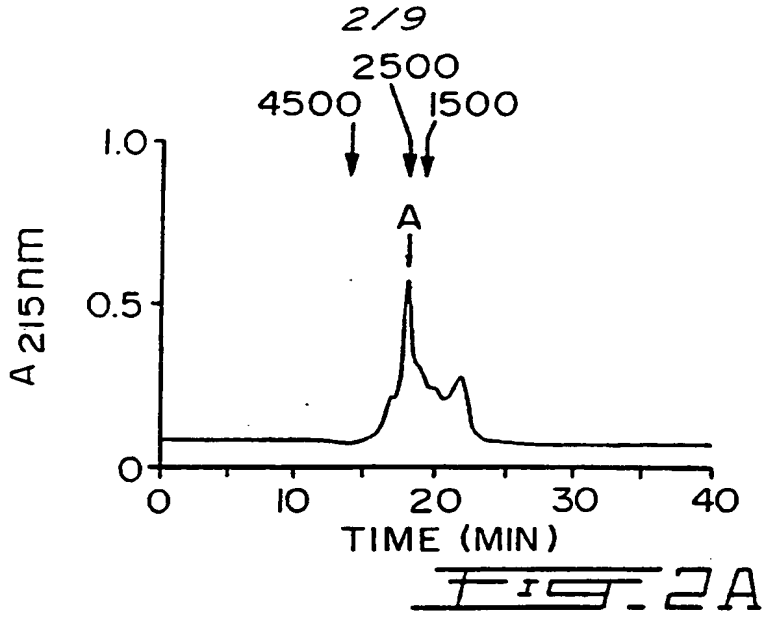

Fig. 2A

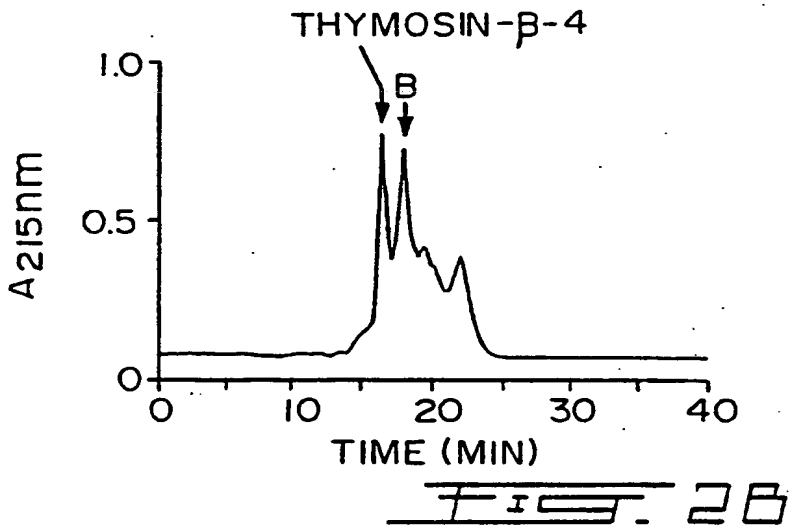

Fig. 2B

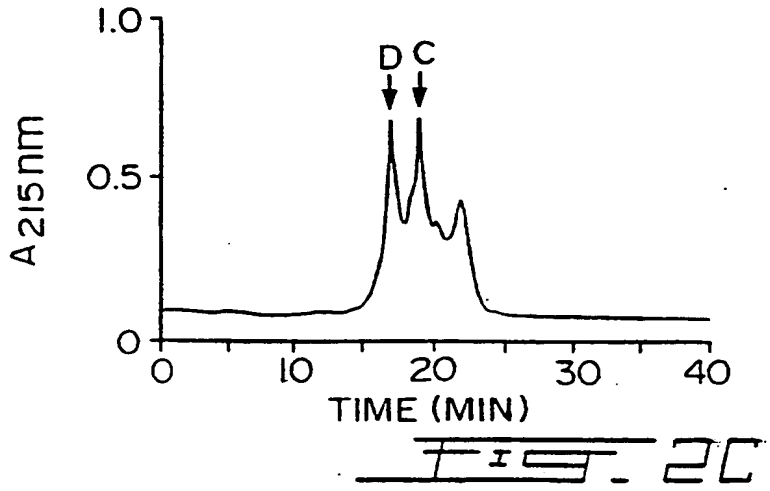

Fig. 2C

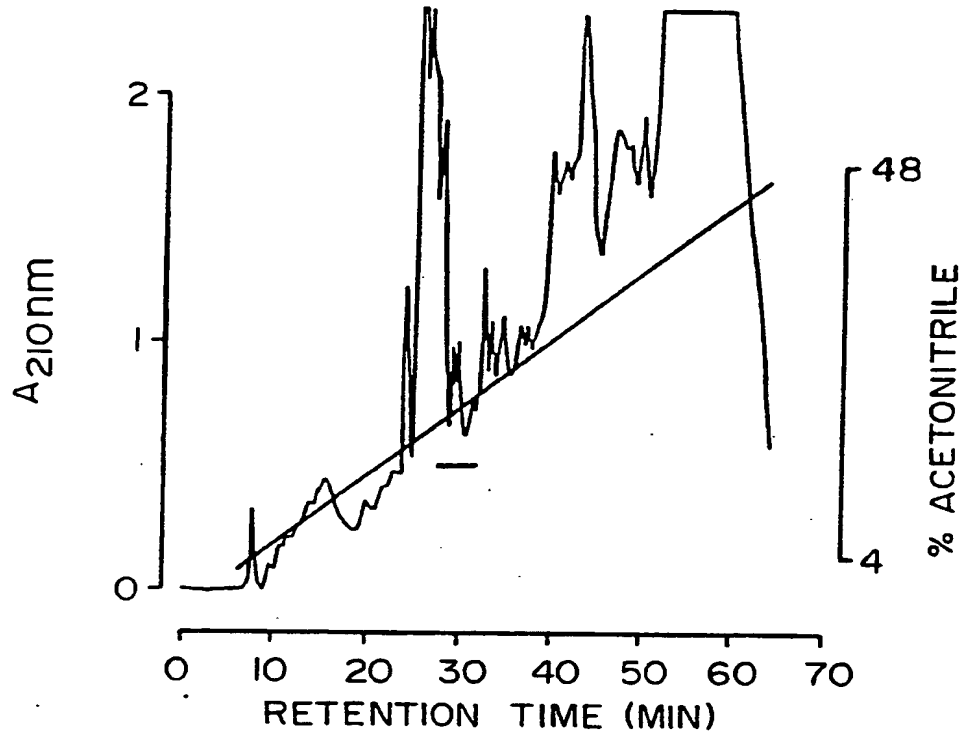
2
1
0
A210nm
48
4
% ACETONITRILE
0 10 20 30 40 50 60 70
RETENTION TIME (MIN)

FIG. 3A

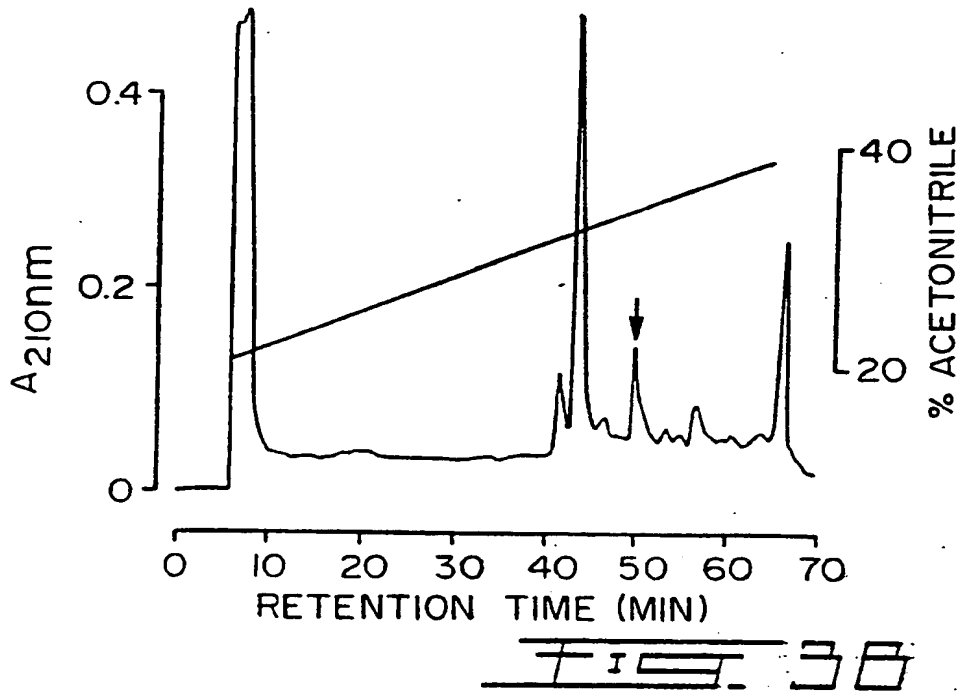
0.4
0.2
0
A210nm
40
20
% ACETONITRILE
0 10 20 30 40 50 60 70
RETENTION TIME (MIN)

FIG. 3B

GATGTGAAGTGTGATATGGAGGTGAGCTGCCCAGATGGCTATACCTGCTGCCGTCTACAGTCGGGG
D V K C D M E V S C P D G Y T C C R L Q S G

GCCTGGGGCTGCTGCCCTTTTACCCAGgtaccaggtgcggcggtggctgagcacagtgtgcagcag
A W G C C P F T Q

ccggccccagtgcccacctgcccttcttcatctgccctagGCTGTGTGTTGTGAAGACCACATCCA
A V C C E D H I H

Fig. 4A

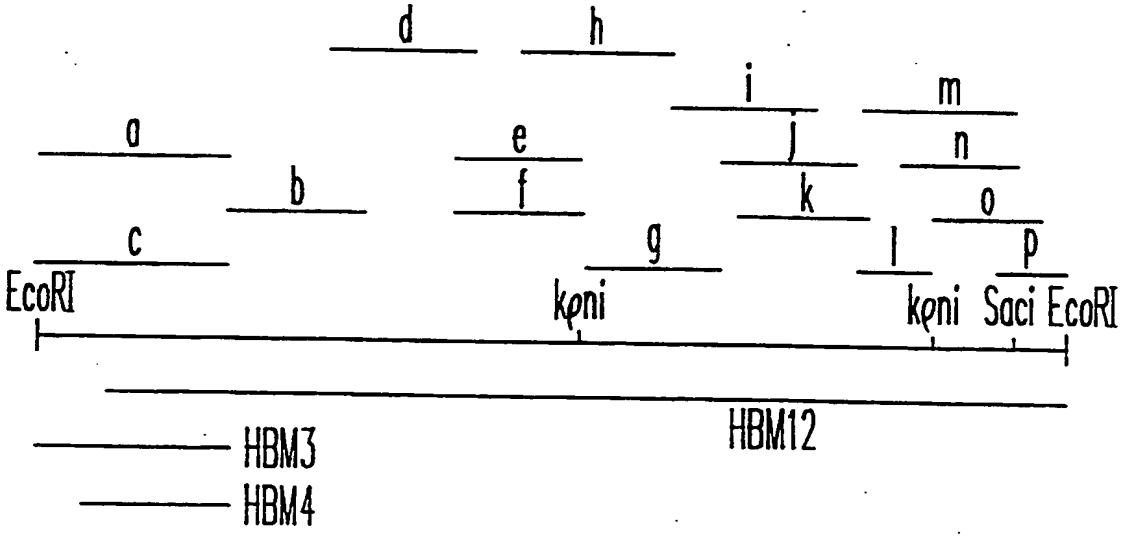

Fig. 4B

```
                    5'... GCGTGGATCCTGAGAACTTCAGGCTCCTGGGCAACGTGCTG
GTCTTGTGTGCTGGCCCATCACTTTGCAAAGAATTCACCCCACCAG]-CGCAGGCAGACC
                                                5'... GCGCGGAGTCGGA

ATGTGGACCCTGGTGAGCTGGGTGGCCTTAACAGCAGGGCTGGTGGCTGGAACGCGGTGC      60
 M  W  T  L  V  S  W  V  A  L  T  A  G  L  V  A  G  T  R  C
                                                   1
CCAGATGGTCAGTTCTGCCCTGTGGCCTGCTGCCTGGACCCCGGAGGAGCCAGCTACAGC     120
 P  D  G  Q  F  C  P  V  A  C  C  L  D  P  G  G  A  S  Y  S
          10                                  20
TGCTGCCGTCCCCTTCTGGACAAATGGCCCACAACACTGAGCAGGCATCTGGGTGGCCCC     180
 C  C  R  P  L  L  D  K  W  P  T  T  L  S  R  H  L  G  G  P
          30                                  40
TGCCAGGTTGATGCCCACTGCTCTGCCGGCCACTCCTGCATCTTTACCGTCTCAGGGACT     240
 C  Q  V  D  A  H  C  S  A  G  H  S  C  I  F  T  V  S  G  T
          50                                  60
TCCAGTTGCTGCCCCTTCCCAGAGGCCGTGGCATGCGGGGATGGCCATCACTGCTGCCCA     300
 S  S  C  C  P  F  P  E  A  V  A  C  G  D  G  H  H  C  C  P
          70                                  80
CGGGGCTTCCACTGCAGTGCAGACGGGCGATCCTGCTTCCAAAGATCAGGTAACAACTCC     360
 R  G  F  H  C  S  A  D  G  R  S  C  F  Q  R  S  G  N  N  S
          90                                  100 *
GTGGGTGCCATCCAGTGCCCTGATAGTCAGTTCGAATGCCCGGACTTCTCCACGTGCTGT     420
 V  G  A  I  Q  C  P  D  S  Q  F  E  C  P  D  F  S  T  C  C
         110                                 120
GTTATGGTCGATGGCTCCTGGGGGTGCTGCCCCATGCCCCAGGCTTCCTGCTGTGAAGAC     480
 V  M  V  D  G  S  W  G  C  C  P  M  P  Q  A  S  C  C  E  D
         130                                 140
AGGGTGCACTGCTGTCCGCACGGTGCCTTCTGCGACCTGGTTCACACCCGCTGCATCACA     540
 R  V  H  C  C  P  H  G  A  F  C  D  L  V  H  T  R  C  I  T
         150                                 160
CCCACGGGCACCCACCCCCTGGCAAAGAAGCTCCCTGCCCAGAGGACTAACAGGGCAGTG     600
 P  T  G  T  H  P  L  A  K  K  L  P  A  Q  R  T  N  R  A  V
         170                                 180
GCCTTGTCCAGCTCGGTCATGTGTCCGGACGCACGGTCCCGGTGCCCTGATGGTTCTACC     660
 A  L  S  S  S  V  M  C  P  D  A  R  S  R  C  P  D  G  S  T
         190                                 200
TGCTGTGAGCTGCCCAGTGGGAAGTATGGCTGCTGCCCAATGCCCAACGCCACCTGCTGC     720
 C  C  E  L  P  S  G  K  Y  G  C  C  P  M  P  N  A  T  C  C
         210                                 * 220
TCCGATCACCTGCACTGCTGCCCCCAAGACACTGTGTGTGACCTGATCCAGAGTAAGTGC     780
 S  D  H  L  H  C  C  P  Q  D  T  V  C  D  L  I  Q  S  K  C
         230                                 240
CTCTCCAAGGAGAACGCTACCACGGACCTCCTCACTAAGCTGCCTGCGCACACAGTGGGC     840
 L  S  K  E  N  A  T  T  D  L  L  T  K  L  P  A  H  T  V  G
            *    250                         260
GATGTGAAATGTGACATGGAGGTGAGCTGCCCAGATGGCTATACCTGCTGCCGTCTACAG     900
 D  V  K  C  D  M  E  V  S  C  P  D  G  Y  T  C  C  R  L  Q
         270                                 280
TCGGGGGCCTGGGGCTGCTGCCCTTTTACCCAGGCTGTGTGCTGTGAGGACCACATACAC     960
 S  G  A  W  G  C  C  P  F  T  Q  A  V  C  C  E  D  H  I  H
         290                                 300
```

FIG. 4C(1)

```
TGCTGTCCCGCGGGGTTTACGTGTGACACGCAGAAGGGTACCTGTGAACAGGGGCCCCAC  1020
 C  C  P  A  G  F  T  C  D  T  Q  K  G  T  C  E  Q  G  P  H
                          310                 320
CAGGTGCCCTGGATGGAGAAGGCCCCAGCTCACCTCAGCCTGCCAGACCCACAAGCCTTG  1080
 Q  V  P  W  M  E  K  A  P  A  H  L  S  L  P  D  P  Q  A  L
                          330                 340
AAGAGAGATGTCCCCTGTGATAATGTCAGCAGCTGTCCCTCCTCCGATACCTGCTGCCAA  1140
 K  R  D  V  P  C  D  N  V  S  S  C  P  S  S  D  T  C  C  Q
                          350 *               360
CTCACGTCTGGGGAGTGGGGCTGCTGTCCAATCCCAGAGGCTGTCTGCTGCTCGGACCAC  1200
 L  T  S  G  E  W  G  C  C  P  I  P  E  A  V  C  C  S  D  H
                          370                 380
CAGCACTGCTGCCCCCAGCGATACACGTGTGTAGCTGAGGGGCAGTGTCAGCGAGGAAGC  1260
 Q  H  C  C  P  Q  R  Y  T  C  V  A  E  G  Q  C  Q  R  G  S
                          390                 400
GAGATCGTGGCTGGACTGGAGAAGATGCCTGCCCGCCGCGGTTCCTTATCCCACCCCAGA  1320
 E  I  V  A  G  L  E  K  M  P  A  R  R  G  S  L  S  H  P  R
                          410                 420
GACATCGGCTGTGACCAGCACACCAGCTGCCCGATGGGCGGAACCTGCTGCCCGAGCCAG  1380
 D  I  G  C  D  Q  H  T  S  C  P  V  G  G  T  C  C  P  S  Q
                          430                 440
GGTGGGAGCTGGGCCTGCTGCCAGTTGCCCCATGCTGTGTGCTGCGAGGATCGCCAGCAC  1440
 G  G  S  W  A  C  C  Q  L  P  H  A  V  C  C  E  D  R  Q  H
                          450                 460
TGCTGCCCGGCTGGCTACACCTGCAACGTGAAGGCTCGATCCTGCGAGAAGGAAGTGGTC  1500
 C  C  P  A  G  Y  T  C  N  V  K  A  R  S  C  E  K  E  V  V
                          470                 480
TCTGCCCAGCCTGCCACCTTCCTGGCCCGTAGCCCTCACGTGGGTGTGAAGGACGTGGAG  1560
 S  A  Q  P  A  T  F  L  A  R  S  P  H  V  G  V  K  D  V  E
                          490                 500
TGTGGGGAAGGACACTTCTGCCATGATAACCAGACCTGCTGCCGAGACAACCGACAGGGC  1620
 C  G  E  G  H  F  C  H  D  N  Q  T  C  C  R  D  N  R  G  G
                          510  *              520
TGGGCCTGCTGTCCCTACGCCCAGGGCGTCTGTTGTGCTGATCGGCGCCACTGCTGTCCT  1680
 W  A  C  C  P  Y  A  Q  G  V  C  C  A  D  R  R  H  C  C  P
                          530                 540
GCTGGCTTCCGCTGCGCACGCAGGGGTACCAAGTGTTTGCGCAGGGAGGCCCCGCGCTGG  1740
 A  G  F  R  C  A  R  R  G  T  K  C  L  R  R  E  A  P  R  W
                          550                 560
GACGCCCCTTTGAGGGACCCAGCCTTGAGACAGCTGCTGTGAGGGACAGTACTGAAGACT  1800
 D  A  P  L  R  D  P  A  L  R  Q  L  L  #
                          570
CTGCAGCCCTCGGGACCCCACTCGGAGGGTGCCCTCTGCTCAGGCCTCCCTAGCACCTCC  1860
CCCTAACCAAATTCTCCCTGGACCCCATTCTGAGCTCCCCATCACCATGGGAGGTGGGGC  1920
CTCAATCTAAGGCCCTTCCCTGTCAGAAGGGGGTTGAGGCAAAAGCCCATTACAAGCTGC  1980
CATCCCCTCCCCGTTTCAGTGGACCCTGTGGCCAGGTGCTTTTCCCTATCCACAGGGGTG  2040
TTTGTGTGTTGGGTGTGCTTTCAATAAAGTTTGTCACTTTCTTAAAAAAAAAAAAAAAA  2099
```

FIG. 4C (III)

```
GRANULIN A      DVKCDMEVS-CPDGYTCCRLQSGAWG-CCPFTQAVCCEDHIHCCPAGFTCDTQKGTCE
GRANULIN B      VMCPDARSRCPDGSTCCELPSGKYG-CCPMPNATCCSDHLHCCPQDTVCDLIQSKCL
GRANULIN C      VPCDNVSS-CPSSDTCCQLTSGEWG-CCPIPEAVCCSDHQHCCPQRYTCVAEGQ-CQ
GRANULIN D       IGCDQHTS-CPVGGTCCPSQGGSWA-CCQLPHAVCCEDRQHCCPAGYTCNVKARSCE
GRANULIN E      DVECGEGHF-CHDNQTCCRDNRQGWA-CCPYAQGVCCADRRHCCPAGFRCARRGTKCL
GRANULIN F      AIQCPDSQFECPDFSTCCVMVDGSWG-CCPMPQASCCEDRVHCCPHGAFCDLVHTRCI
GRANULIN G      GGPCQVDAH-CSAGHSCCIFTVSGTSS-CCPFPEAVACGDGHHCCPRGFHCSADGRSCF
PARAGRANULIN     TRCPDGQF-CPVA--CCLDPGGASYSCCRPLLD
```

FIG. 5

Fig. 6

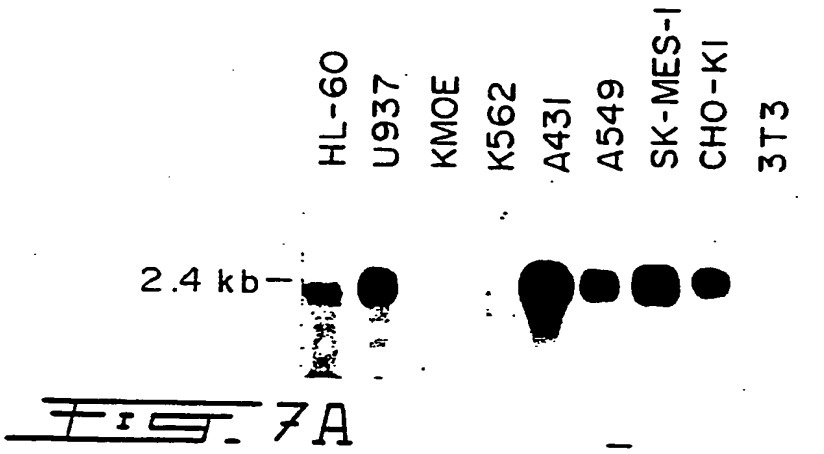

Fig. 7A

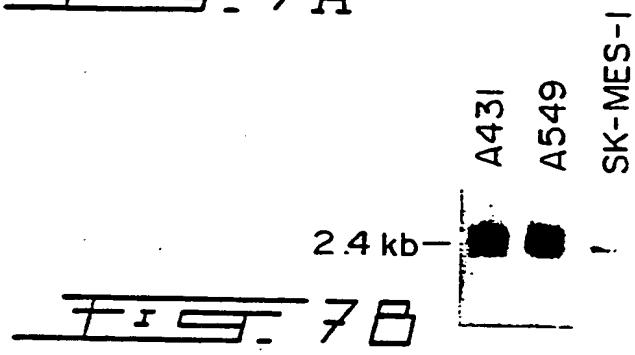

Fig. 7B

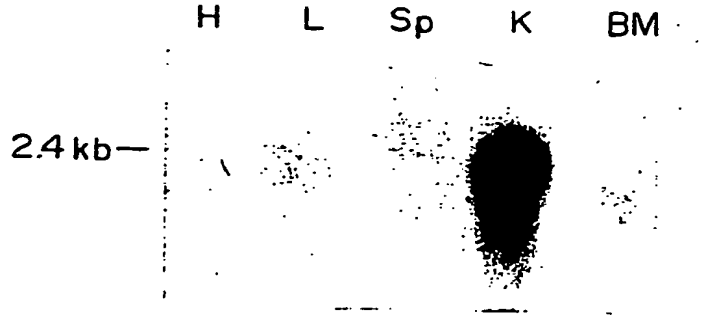

Fig. 7C

INTERNATIONAL SEARCH REPORT

International Application No PCT/CA 92/00089

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 12 N 15/12 C 07 K 7/10 C 07 K 15/06
A 61 K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 07 K C 12 N A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS vol. 173, no. 3, 31 December 1990, DULUTH, MINNESOTA US pages 1161 - 1168 BATEMAN, A. ET AL. 'Granulins, a novel class of peptide from leukocytes' cited in the application see the whole document ---	1-2,12-14
X	WO,A,9115510 (BRISTOL-MYERS SQUIBB COMPANY) 17 October 1991 see the whole document, especially figure 22 and the claims --- -/-	1-2,12-14

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
21-09-1992	05. 03 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	S.M. ANDRES

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 87, no. 20, October 1990, WASHINGTON US pages 7912 - 7916 SHOYAB, M. ET AL. 'Epithelins 1 and 2: Isolation and characterization of two cysteine-rich growth-modulating proteins' cited in the application see the whole document	1-2,12-14

INTERNATIONAL SEARCH REPORT

I national application No.
PCT/CA92/00089

Box I Observations where certain claims were found unsearchable (Continuati n f item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see PCT/ISA/206 mailed on 26.10.92

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

(1-2) completely, (12-14) partially

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. CA 9200089 SA 56884

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 04/01/93
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 9115510	17-10-91	AU-A- 7744791	30-10-91
		CN-A- 1058782	19-02-92